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COMB-LIKE SOLID PHASE FOR MEASURING ANALYTES

FIELD OF THE INVENTION

The present invention relates generally to the field of biological and biochemical assays.

BACKGROUND OF THE INVENTION

It is of great importance in all fields and disciplines of the life sciences to utilize qualitative and quantitative analytical techniques for the detection, identification, and measurement of a wide variety of biologically important molecules. For example, these analytical techniques can be utilized in many different types of assays including those for enzymes, receptors, lectins and inhibitors.

As virtually all chemical reactions in living systems are catalyzed by enzymes, the assay of enzyme activity is one of the most frequently encountered procedures in biochemistry. Typically, most enzyme assays are used to estimate the amount or activity of an enzyme present in a cell, tissue, other preparation, or as an essential part of an enzyme purification protocol. The current assay methods have been developed based on the physical, chemical and immunological properties of the enzyme of interest and use detection means such as for example photometric, radiometric, high performance liquid chromatographic, and electrochemical assays. (Eisenthal, R. and Danson, M. J., in *Enzyme Assays: a practical approach*. (IRL Press: Oxford University, Oxford, (1993)). Although the above-described methods basically fulfill the requirements for routine analysis, there are the disadvantages of low sensitivity (Oppert et al, 1997, *BioTechniques* **23**:70-72), multiple steps (Twining, S. S., 1984 *Anal Biochem* **143**: 30-344; Pazhanisamy, S. et al, 1995, *Anal Biochem* **229**: 48-53) and steps that are tedious and time-consuming (Fields, R.,. 1972, *Meth Enz* **25B**: 464-468).

Similarly, immunoassays have been widely using in human clinical tests and therapeutics, agriculture, food, veterinary and environmental diagnostics (Deshpandes, S. in *Enzyme immunoassays from concept to product development*., (Chapman & Hall: New York, 1996)). While for most purposes, immunoassays are effective (Cleaveland, J. S. et al, 1990, *Anal Biochem* **190**: 249-253.), in some cases they are not suitable, for example, in the determination of enzyme activity. This is due to the fact that binding assays for antibody and antigen (in this instance, an enzyme)

only measure the concentration of an antigen (enzyme) and not its activity. Specifically, it is often important to know the catalytic activity of an enzyme and not just the concentration of the enzyme as a given amount of enzyme may have varying activity depending on reaction conditions, source of enzyme and the isolation protocol used. Also, antibodies tend to react only with structurally similar antigens. Therefore, it is often not possible to quantitate the amount of an enzyme from a given species using specific antibodies from a related species using immunoassays.

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The pharmaceutical industry utilizes the methods mentioned above to screen compounds for discovering drugs. This process is slow due to the multiple steps required and the large amount of compounds needed to be tested. Typically, on a good day, a lab might test 100 to 1,000 compounds. However, in the race to commercialize, pharmaceutical manufacturers are facing great pressure to reduce the time required to discover new clinical drugs, cut assay costs, and screen more compounds and against more targets. Therefore, there is a very high demand to develop new methods to meet the requirements of High Throughput Screening (HTS). There has been described a method using quenched BODIPY dye-labeled casein as a substrate for determining the activities of protease, which is sensitive and amenable to automation (Jones, L. J. et al, 1997, *Anal Biochem* **251**: 144-152). The degree of quenching of the fluorescent tag is crucial in this method. However, if there is not enough quenching due to poor conjugation or degradation of the fluorescence-labeled substrate under storage, etc. the assay will not be very useful. Also this procedure has relatively high background values which reduce its sensitivity. Another example of a potentially useful high throughput assay was made by Marquardt, et al and described in PCT/WO97/43438. The method involves many steps, including coating the wells of a microplate, washing the wells, adding biologically active substance to the wells, washing the wells once more, adding the indicator enzyme to the wells, washing the wells again and adding a color development reagent. As a result, the assay cannot be readily used in assays requiring rapid analysis.

Clearly, a new assay method that is highly sensitivity and suitable for high throughput assays is needed. Specifically, the assay should be suitable for a wide variety of applications, easy to use and require only a few steps for obtaining

results.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a new assay method that is highly sensitive and suitable for high throughput assays.

This invention provides a new method for the qualitative and quantitative analysis of bioactive substances. The assay method is based on a one-step procedure for separating the reactants from the products (resultants) after completion of the reaction followed by measurement of the amount of a labeled reactant or labeled product that has been left in the reaction vessel or on the pin. The device consists of two parts: one of which is a reaction vessel and other is a pin-like device (a probe) that can fit into the reaction vessel. The reaction begins by insertion of the pin-like device that is coated with a reactant into the reaction vessel containing the same or other reactants than those coated on the pin-like surface. The said pin-like surface is taken out from the reaction vessel to stop the reaction. The amount of the labeled products or labeled reactant remaining in the reaction vessel or on the probe can be determined according to the intensity of its label which can be fluorescent, luminescent and chromogenic molecules or radioactive tag, etc. The amount of the label in the reaction vessel is directly or reciprocally proportional to the activity or amount of the bioactive substance that is to be measured. The device can be used for the assay of enzymes, their inhibitors, antibodies and antigens, and receptors or lectins and their ligands.

According to a first aspect of the invention, there is provided a method for measuring the activity or concentration of a biomolecule comprising:

providing a reaction vessel containing a sample, said sample including a biomolecule having a biological activity;

providing a probe coated with a reactant, said reactant being capable of interacting with the biomolecule;

adding a known quantity of a compound with a detectable label to the sample;

inserting the probe into the reaction vessel such that the biomolecule and the detectable label contact the reactant and interact with the reactant such that

label is bound to either the reactant or the biomolecule;

removing the probe from the reaction vessel; and

measuring the quantity of detectable label in the reaction vessel and/or on the probe.

The probe may have a shape selected from the group consisting of: pin-like; cone-like; cuboid; cylindrical; star-shaped; and spire-shaped.

The detectable label may be selected from the group consisting of: colorimetric label; radioactive label; luminescent label; and fluorescent label.

The sample may be a biological sample.

The biological activity may be an enzyme; a receptor; a lectin or an antibody; or a specific ligand of an antibody, a receptor, a lectin or an enzyme.

The reactant may be bound to the probe.

The sample may include an inhibitor of the biological activity of the biomolecule.

The sample may include a competitor of the biological activity of the biomolecule.

The biomolecule may be selected from the group consisting of: an enzymatic product; an enzyme; a substrate; a receptor; an inhibitor; a ligand; a lectin; a lectin-binding ligand; a receptor-binding ligand; an antibody; an antigen; a receptor; and a lectin.

The compound may be selected from the group consisting of: an enzymatic product; an enzyme; a substrate; a receptor; an inhibitor; a ligand; a lectin; a lectin-binding ligand; a receptor-binding ligand; an antibody; an antigen; a receptor; and a lectin.

According to a second aspect of the invention, there is provided a method for measuring the activity or concentration of a biomolecule comprising:

providing a reaction vessel containing a sample, said sample including a biomolecule having a biological activity;

providing a probe coated with a reactant, said reactant being capable of interacting with the biomolecule, said reactant including a detectable label;

inserting the probe into the reaction vessel such that the reactant and

the detectable label contact the biomolecule and interact with the biomolecule such that label is released from the reactant;

removing the probe from the reaction vessel; and

measuring the quantity of detectable label in the reaction vessel and/or on the probe.

The probe may have a shape selected from the group consisting of: pin-like; cone-like; cuboid; cylindrical; star-shaped; and spire-shaped.

The detectable label may be selected from the group consisting of: colorimetric label; radioactive label; luminescent label; and fluorescent label.

The reactant may be bound to the probe.

The sample may be a biological sample.

The biological activity may be an enzymatic activity or a binding affinity.

The sample may include an inhibitor of the biological activity of the biomolecule.

The sample may include a competitor of the biological activity of the biomolecule.

The biomolecule may be selected from the group consisting of: an an enzymatic product; an enzyme; a substrate; a receptor; an inhibitor; a ligand; a lectin; a lectin-binding ligand; a receptor-binding ligand; an antibody; an antigen; a receptor; and a lectin.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

DEFINITIONS

"Ligand" as used herein refers to a bioactive molecule having specific binding affinity for another biomolecule.

"Receptor" as used herein refers to a bioactive molecule that has a specific binding affinity for another biomolecule, for example, a ligand.

"Bioactive molecule" or "biologically active substance" as used herein except where otherwise stated refers to a molecule or complex having a biological activity, for example, an enzymatic activity or binding affinity for another biomolecule.

"Probe" as used herein refers to a member arranged to be reversibly inserted into a reaction mixture, as discussed below.

The present invention relates to a method for the detection, identification and measurement of the amount or activity of biologically active molecules via a one-step separation of reactants from products using a device comprising a probe whose surface is coated with a biologically active substance and a reaction vessel containing the reaction mixture. The reaction mixture contains a bioactive molecule and may, for example, be a biological sample or a synthetic reaction mixture in which all of the components are known.

As discussed above, the assay consists of a probe and a reaction vessel. As discussed above, the reaction vessel contains a reaction mixture which includes a bioactive molecule, for example, an enzyme or ligand, as well as possibly other compounds, for example, inhibitors, antibodies or receptors, as described below. The probe has a surface and the surface of the probe is coated with a compound. It is of note that the compound selected for coating is either a substrate for or has binding affinity for the bioactive molecule, as described below. Furthermore, the compound may be labeled, for example, fluorescently, luminescently, colorimetrically or radioactively, or the reaction mixture may contain a labeled substrate for or a labeled biomolecule having binding affinity for the bioactive molecule. In the first instance, wherein the compound is labeled, the labeled group on the compound is selected such that the activity of the bioactive molecule causes the label to be cleaved or removed from the compound and released to the reaction mixture. In the second instance, the label on the substrate within the reaction mixture is selected such that the activity of the bioactive molecule will cause the label to be transferred from the labeled substrate to the compound coating the surface of the probe, as described below. Alternatively, the labeled biomolecule and the

biomolecule in the reaction mixture may compete for binding to the compound on the surface of the probe, or the biomolecule in the reaction mixture may compete with the labelled compound in the reaction mixture and the compound on the surface of the probe, as described below.

In general, the assay is carried out as follows: the quantity of labeled compound coated on the surface of the probe or the quantity of labeled substrate in the reaction vessel may first be determined by means known in the art according to the type of label used. The probe is then lowered into the reaction vessel such that the coated surface of the probe contacts the reaction mixture. The bioactive molecules present in the reaction mixture then either release label from the compound, transfer label from the substrate in the reaction mixture to the compound on the surface of the probe or compete for binding, as described below. Once the probe has been in contact with the reaction mixture for a pre-determined period of time, the probe is removed from the reaction mixture, thereby terminating the reaction without the need to add additional chemicals, heat the reaction mixture or centrifuge the reaction mixture. The quantity of label either in the reaction mixture or on the surface of the probe can then be determined, which can then be used to calculate reaction kinetics.

In the embodiment described herein, the probe has a substantially pin-like shape. It is of note that in other embodiments, the probe may be of other shapes, for example, cylindrical, spire-shaped, star-shaped, cuboid, or cone-shaped.

This is in contrast with traditional methods, which, for example, may involve providing a reaction vessel containing a reaction mixture including a bioactive molecule, adding a labeled compound that is a substrate for the bioactive molecule, stopping the reaction, that is, stopping the biological activity of the bioactive molecule, and separating unused substrate from used substrate by washing or chromatography. An alternative type of assay known in the art involves coating a reaction vessel with a compound, adding a ligand, allowing binding between the ligand and the compound to occur, washing away unbound ligand, and adding a reporter compound to detect bound ligand.

Clearly, these methods are less desirable than the above-described

assay, as they require multiple steps and include washing and/or separation steps which can cause considerable inaccuracy in experimental results. However, the above-described assay does not require washing or separation steps and involves relatively few steps, resulting in shorter assay times, more reliable results and greater ease of use.

The invention will now be described using examples. Specifically, the examples provide broad examples of methods of using the assay as well as some specific examples. However, the scope of the invention is not limited to these examples

EXAMPLE I – TRANSFER OF LABEL FROM THE REACTION MIXTURE TO THE PROBE

For example, the surface of the probe, in this embodiment a pin-like device, is coated with reactant 1 which is an acceptor of a moiety from reactant 3. Reactant 3 is a substrate for reactant 2 having a biological activity. That is, in this example, reactant 1 is the compound used to coat the probe, reactant 3 is the labeled substrate and reactant 2 is the bioactive molecule. Furthermore, reactant 2 and reactant 3 are present in the reaction mixture in the reaction vessel. When the coated pin-like device is inserted into a reaction vessel, the labeled moiety from reactant 3 is transferred to reactant 1 due to the biological activity of reactant 2 acting on reactants 1 and 3. The amount of labeled reactant 3 remaining in the reaction vessel can be directly determined without an additional step and is reciprocally proportional to the activity of reactant 2 after the pin-like device containing the labeled moiety from reactant 3 has been taken out of the reaction vessel. Alternatively, the amount of label transferred to the probe may be determined, which is directly proportional to the activity of reactant 2.

EXAMPLE II - TRANSFER OF LABEL FROM THE REACTION MIXTURE TO THE PROBE IN PRESENCE OF INHIBITOR

In this example, the transfer of the labeled moiety between reactant 1 and reactant 3 by reactant 2 is interfered with by reactant 4 which is an inhibitor of reactant 2. Thus, when the coated probe is inserted into the reaction vessel, reactant 4 inhibits transfer of the labeled moiety from reactant 3 to reactant 1.The amount of

labeled reactant 3 remaining in the reaction vessel once the probe has been removed from the reaction vessel is directly proportional to the amount of reactant 4 (inhibitor) in the reaction mixture. Similarly, the amount of labeled reactant 1 present on the surface of the probe is reciprocally proportional to the amount of reactant 4 (inhibitor) present in the reaction mixture.

EXAMPLE III – TRANSFER OF LABEL FROM THE PROBE TO THE REACTION MIXTURE

In this example, the surface the probe, in this embodiment, a pin-like device is coated with reactant 1 which is a labeled substrate for reactant 2 having a biological activity. That is, in this example, reactant 1 is the labeled compound and reactant 2 is the bioactive molecule. Insertion of the pin-like device containing reactant 1 into a reaction vessel containing reactant 2 and other compounds essential for the reaction results in the initiation of a reaction in which labeled products of the reaction are released into the reaction vessel. The amount of the released label in the reaction vessel can be directly measured after the pin-like device has been taken out of the reaction vessel without an additional step and the quantity of label released is proportional to the activity of reactant 2. Alternatively, as discussed above, the amount of label remaining on the pin-like device can be determined, which would be reciprocally proportional to the activity of reactant 2.

EXAMPLE IV – TRANSFER OF LABEL FROM THE PROBE TO THE REACTION MIXTURE IN THE PRESENCE OF AN INHIBITOR

In this example, the release of label from reactant 1 coated on the surface of the pin-like device by reactant 2 is interfered with by reactant 3 which is an inhibitor of reactant 2 and is present in the reaction mixture. Insertion of the pin-like device containing reactant 1 into a reaction vessel containing reactant 2 and other compounds essential for the reaction as well as reactant 3, the inhibitor, results in the initiation of a reaction in which labeled products of the reaction are released into the reaction vessel. The amount of the released label from reactant 1 to the reaction vessel is reciprocally proportional to the amount of reactant 3 (inhibitor) after the pin-like device has been taken out of the reaction vessel. Alternatively, as discussed above, the amount of label remaining on the pin-like device can be determined,

which would be directly proportional to the activity of reactant 3 (inhibitor) on reactant 2 (bioactive molecule).

EXAMPLE V – CAPTURE OF ENZYMATIC PRODUCT BY THE PROBE

In this example, the surface of a pin-like device is coated with reactant 1 which is a binding ligand for product 1. Product 1 is formed from reactant(s) 3 linked with either a label or a binding agent capable of binding specifically to reactant 1. That is, product 1 is caused by the biological activity of reactant 2 in a reaction vessel acting on reactant(s) 3. The coated pin-like device is then inserted into the reaction vessel containing product 1 and other non-reacted reactants. Thus, the probe is coated with a compound (reactant 1) that binds or has binding affinity for a second compound (product 1). This second compound (product 1) is formed through the action of the bioactive molecule (reactant 2) and components of the reaction mixture (reactant(s) 3). Thus, as product 1 is formed through the action of reactant 2, product 1 binds to reactant 1 which coats the surface of the probe. After the pin-like device has been taken out of the reaction vessel, the amount of labeled non-reacted reactant 3 remaining in the reaction vessel can be directly measured without an additional step and is reciprocally proportional to the activity of reactant 2. Alternatively, the amount of labeled product 1 bound to the probe can be directly measured, which is directly proportional to the activity of reactant 2.

EXAMPLE VI – CAPTURE OF ENZYMATIC PRODUCT BY THE PROBE IN THE PRESENCE OF INHIBITOR

In this example, the formation of product 1 from reactants 3 due to biological activity of reactant 2 is interfered with by reactant 4 which is an inhibitor of reactant 2. The assay is carried out as described above (Example V), except that in this instance, the amount of labeled non-reacted reactant 3 remaining in the reaction vessel after the pin-like device has been taken out of the reaction vessel is directly proportional to the amount of reactant 4 (inhibitor) present in the reaction mixture. Alternatively, the amount of labeled product 1 bound to the probe can be directly measured, which is reciprocally proportional to the amount of reactant 4 (inhibitor) present in the reaction mixture.

EXAMPLE VII – COMPETITIVE BINDING

In this example, the surface of a pin-like device is coated with reactant 1 which is a binding ligand for reactant 3 being the labeled form of reactant 2 or a labeled compound that will compete for binding with reactant 2 for binding to reactant 1, as described below. Alternatively, reactant 3 may be chosen such that reactant 1 and reactant 2 will compete for binding to reactant 3, as described below. Competition between reactant 2 and reactant 3 for binding to reactant 1 or between reactant 2 and reactant 1 for binding to reactant 3 are initiated when the pin-like device bound using reactant 1 is inserted into a reaction vessel containing a unknown amount of reactant 2 and a known amount of labeled reactant 3. That is, the probe is coated with a first compound and the reaction vessel contains an unknown quantity of a second compound. A known quantity of a labeled third compound is added to the reaction vessel and the coated probe is inserted into the reaction mixture. Specifically, the labeled third compound is selected such that the third compound competes with the second compound for binding to the first compound or the first compound and the second compound compete for binding of the labeled third compound. The amount of the labeled reactant 3 remaining in the reaction vessel can be directly measured without an additional step after the pin-like device has been taken out of the reaction vessel and is directly proportional to the amount of reactant 2 in the reaction vessel. Alternatively, the amount of labeled product 1 bound to the probe can be directly measured, which is reciprocally proportional to the amount of reactant 2 in the reaction vessel. Thus, in the first instance labeled reactant 3 competes with reactant 2 for binding to the coated probe. In the second instance, reactant 1 and reactant 2 compete for binding to the labeled third compound. However, in both instances, the relative amount of reactant 3 bound to the probe or remaining in the reaction vessel provides information on the amount of reactant 2 in the reaction vessel.

EXAMPLE VIII – COMPETITIVE BINDING IN PRESENCE OF INHIBITOR

In this example, the reactions of the competitive binding between reactant 2 and reactant 3 for reactant 1 or between reactant 2 and reactant 1 for reactant 3 as described above are interfered with by reactant 4 which is an inhibitor of reactant 2. The amount of the labeled reactant 3 remaining in the reaction vessel

after the pin-like device has been taken out of the reaction vessel is directly proportional to the amount of reactant 4 (inhibitor) in the reaction vessel.

EXAMPLE IX – PROTEIN KINASE A ASSAY

In this example, an assay of protein kinase A activity in the presence and absence of its inhibitor using the above-described assay is described. As will be seen, this is an example of the assay described in Examples I and II, that is, the transfer of label from the reaction mixture to the probe in the presence and absence of inhibitor.

Materials: hydrolyzed and partially dephosphorylated casein, protein kinase 3' : 5'-cyclic AMP dependent (PKA), cyclic AMP, protein kinase inhibitor, Na_2HPO_4, NaH_2PO_4, NaCl, Tween-20 are from Sigma. ^{32}P-ATP is from New England Nuclear. The 96-well microplate and 96-pin lid are from VWR Canlab.

Method:

1. Preparation of a coated 96-pin lid:

(1). 5 g skim milk powder dissolved in 100 ml phosphate buffer saline (PBS, pH 7.2) is added to the wells of a microplate and incubated at 37^0 C for 2 hr. The microplate is washed three times with PBST (PBS + Tween).

(2). Casein is dissolved in PBS to the concentration 5 ug/ml and 100 ul/well is added to each well of the microplate blocked with skim milk. The 96-pin lid is inserted into the wells of the microplate and incubated at 37^0 C for 3 hr, and is then taken out and rinsed with PBST. As a result of this arrangement, the probe is coated with casein.

2. Protein kinase assay:

(1). A series of concentrations of protein kinase in phosphate buffer (PB pH 7.2, cAMP, ^{32}P-ATP) are added to the wells of a microplate (100 ul/well).

(2). The reaction is initiated by inserting the 96-pin lid coated with casein. During incubation at 37^0 C for 30 min, ^{32}P is transferred from ^{32}P-ATP to casein by protein kinase. The 96-pin lid is then taken out of the wells of the microplate to stop the reaction.

(3). The radioactivity of ^{32}P-ATP remaining in the wells of the microplate is counted in a scintillation counter and is reciprocally proportional to the activity of the

protein kinase.

3. Protein kinase inhibitor assay:

(1). Varying concentrations of an inhibitor in the phosphate buffer (PB pH 7.2, cAMP, ^{32}P-ATP) are added to the wells (50 ul/well) of a microplate. Negative and positive controls are also included.

(2). A fixed amount of protein kinase A (50 ul/well) is added to each well containing inhibitor and the controls. Inhibition of protein kinase activity by the inhibitor will occur.

(3). The reaction of phosphate transferring is initiated by inserting the 96-pin lid coated with casein followed by incubation at 37^0 C for 30 min. Protein kinase catalyzes the transfer of ^{32}P from ^{32}P-ATP to casein with the degree of transfer being reciprocally related to the concentration of the inhibitor. The 96-pin lid is taken out of wells of the microplate to end the reaction.

(4). The radioactivity of ^{32}P-ATP remaining in the wells of the microplate is counted in a scintillation counter and is directly proportional to the amount of the inhibitor.

EXAMPLE X - FLUORESCENT ASSAY OF PROTEASE AND PROTEASE INHIBITORS

In this example, the activity of a variety of proteases and protease inhibitors are examined using the above-described assay. As will be seen, this is an example of the general assay given in Examples III and IV, wherein the label is transferred from the probe to the reaction mixture.

Materials: proteinase K, elastase, protease XIII, papain, trypsin, pepsin, casein, dimethyl sulfoxide (DMSO), Na_2HPO_4, NaH_2PO_4, NaCl, Tween-20, citrate, ovomucoid, aprotinin are from Sigma. NHS-coumarin is from Molecular Probes. The 96-well microplate and 96-pin lid are from VWR Canlab.

Method:

1. Preparation of a fluo-casein: 5 mg NHS-coumarin in 100 ul DMSO is mixed with 10 mg casein in PBS (pH 7.2) in a microcentrifuge tube and incubate at room temperature for 3 hr.

2. Preparation of a coated 96-pin lid:

(1). 5 g skim milk powder dissolved in 100 ml PBS (pH 7.2) is added to the wells of a microplate and incubated at 37^0 C for 2 hr. The microplate is washed three times with PBST.

(2). Fluo-casein is dissolved in PBS to the concentration 5 ug/ml and 100 ul/well is added to each well of the microplate blocked with skim milk. The 96-pin lid is inserted into the wells of the microplate, incubated at 37^0 C for 3 hr, then taken out and rinsed with PBST.

3. Protease activity assay:

(1). A series of concentrations of a protease in a buffer (100 ul/well) are added to the wells of a microplate.

(2). The reactions are initiated by inserting the 96-pin lid coated with the fluo-casein and incubated at 37^0 C or room temperature for 30 min. The fluo-casein is hydrolyzed by protease to release the fluorescent labels into the reaction vessel. The 96-pin lid is taken out of the reaction vessel to stop the reaction.

(3). The fluorescent intensity of the label in the wells of the microplate is measured with a fluorometer and is directly proportional to the activity of the protease.

4. Protease inhibitor assay:

(1). Varying amounts of the protease inhibitor in the buffer (50 ul/well) are added to the wells of a microplate. Negative and positive controls are included.

(2). A fixed concentration of the protease in the buffer (50 ul/well) is added to the wells containing inhibitor and the controls. The inhibition of the protease by the inhibitor reduces the amount of label released into the reaction mixture.

(3). Hydrolysis of the casein by the protease is initiated by inserting the 96-pin lid coated with fluo-casein. The sample is incubated at 37^0 C or room temperature for 1 hr. The fluo-casein is cleaved by the residual activity of the protease after inhibition to release the fluorescent label into the reaction vessels. The 96-pin lid is taken out of the reaction vessel to stop the reaction.

(4). The fluorescent intensity of the label in the wells of the microplate is measured with a fluorometer and is reciprocally proportional to the amount of the inhibitor.

EXAMPLE XI – TELOMERASE ASSAY

In this example, telomerase activity, in the presence and absence of inhibitor is assayed. As will be seen, this is an example of Examples V and VI, binding of a synthesized product to the probe. Specifically, telomerase produces a nucleic acid molecule using labeled nucleotides and a primer that includes a binding agent which binds to the compound coating the probe. Thus, in this example, unextended primer will bind to the probe but will not produce a signal and unincorporated nucleotides will not be able to bind the probe and will remain in the reaction mixture.

Materials: Tris-acetate buffer (pH 8.5), potassium acetate, β-mercaptoethanol, spermidine, $MgCl_2$, EDTA, streptavidin are from Sigma. dATP, dTTP, fluo-dGTP, telomerase S10, RNase,
biotin-oligonucleotide primer may be purchased from Boehringer Mannheim or elsewhere. Inhibitor (7-deaza-dATP).

Method:

1. Preparation of a coated 96-pin lid:

(1). 5 g skim milk powder dissolved in 100 ml PBS (pH 7.2) is added to the wells of a microplate and incubated at 37^0 C for 2 hr. The microplate is washed three times with PBST.

(2). Streptavidin or avidin is dissolved in a buffer to the concentration 5 ug/ml and 100 ul/well is added to each well of the microplate blocked with skim milk. The 96-pin lid is inserted into the wells of the microplate and incubated at 37^0 C for 3 hr, and is then taken out and rinsed with PBST.

2. Telomerase activity assay:

(1). A series of concentration of telomerase in a reaction mixture containing 50 mM Tris-acetate pH 8.5, 50 mM potassium acetate (KAc), 5 mM β-mercaptoethanol, 1mM spermidine, 1mM $MgCl_2$, 0.5-2 mM dATP, 0.5-2 mM dTTP, 1.5 uM fluo-dGTP, 1 uM biotin-oligonucleotide primer $(TTAGG)_3$, are added to wells of a microplate and the mixture is incubated at 30^0 C for 1 hr. The reaction of DNA synthesis is stopped by adding the stop solution (10 mM Tris-HCl, pH7.5, 230 mM EDTA and 100 ug/ml RNase) at 37^0 C for 15 min.

(2). Isolation of the fluorescent label-DNA with biotin-primer (fluo-DNA-biotin) from the reaction vessel is done by the binding of the fluo-DNA-biotin complex to the streptavidin (avidin)-coated 96 pin lid after the 96-pin lid has been inserted into the reaction vessel.

(3). The fluorescent intensity of fluo-dGTP remaining in the reaction vessel is measured with a fluorometer after the 96-pin lid has been taken out of the reaction vessel and is reciprocally proportional to the activity of telomerase.

3. Telomerase inhibitor assay:

(1). Varying amounts of the inhibitor in the reaction mixture (50 ul/well) are added to the wells of a microplate.

(2). A fixed activity of telomerase in the reaction mixture (50 ul/well) is added to the wells containing an inhibitor and the controls. Incubate the wells of the plate at 30^0 C for 1-2 hr and stop the DNA synthesis with the stop solution.

(3). Isolation of the fluorescent label-DNA with biotin-primer (fluo-DNA-biotin) from the reaction vessel is done via the binding of fluo-DNA-biotin complex to the streptavidin (avidin)-coated 96 pin lid after the 96-pin lid has been inserted into the reaction vessel.

(4). The fluorescent intensity of fluo-dGTP remaining in the reaction vessel is measured with a fluorometer after the 96-pin lid has been taken out of the reaction vessel and is directly proportional to the amount of the inhibitor.

EXAMPLE XII – COMPETITIVE BINDING ASSAY

In this example, competitive binding of E. coli K88 fimbriae, receptor and inhibitor is assayed using the above-described method. This assay is a specific example of the general assay given in Examples VII and VIII, that is, of competitive binding assays using the above-described assay method.

Materials: Na_2HPO_4, NaH_2PO_4, NaCl, dimethyl sulfoxide (DMSO) are from Sigma. NHS-fluorescein is from Molecular Probes. A mucus receptor from a piglet and E. coli K88 fimbriae are prepared in a laboratory. Inhibitors are from different sources.

Method:

1. Preparation of fluo-fimbriae: 5 mg NHS-fluorescein in 100 ul DMSO is mixed with

10 mg fimbriae in 1 ml PBS (pH 7.2) followed by incubation at room temperature for 3 hr.

2. Preparation of a coated 96-pin lid

(1). 5 g skim milk powder dissolved in 100 ml PBS (pH 7.2) is added to the wells of a microplate and incubated at 37^0 C for 2 hr. The microplate is washed three times with PBST.

(2). Receptor is dissolved in PBS (pH 7.2) to a concentration of 5 ug/ml and 100 ul/well is added to each well of the microplate blocked with skim milk. The 96-pin lid is inserted into the wells of the microplate and incubated at 37^0 C for 3 hr, and is then taken out and rinsed with PBST.

3. Receptor assay:

(1). A series of concentrations of the receptor in a buffer (50 ul/well) are added to the wells of a microplate.

(2). A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the receptor and the control.

(3). Competitive binding reactions between the immobilized receptor and the free receptor (competitor) to the fluo-fimbriae are initiated by inserting the 96-pin lid coated with the receptor into the wells. Incubate at 37^0 C for 1 hr . Then the 96-pin lid is taken out of the vessel to stop the reaction.

(4). The fluorescent intensity of the fluo-fimbriae remaining in the wells of the microplate is determined using a fluorometer and is directly proportional to the amount of the receptor (competitor).

4. Fimbriae or E. coli numeration assay:

(1). A series of concentration of the fimbriae or E. coli cell in a buffer (50 ul/well) are added to the wells of a microplate.

(2). A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the fimbriae or E. coli and control wells.

(3). Competitive binding reactions between the fimbriae or E. coli (competitor) and the fluo-fimbriae to the immobilized receptor are initiated by inserting the 96-pin lid coated with the receptor into the wells. Incubate at 37^0 C for 1 hr. Then the 96-pin lid is taken out of the vessel to stop the reaction.

(4). The fluorescent intensity of the fluo-fimbriae remaining in the wells of the microplate is determined using a fluorometer and is directly proportional to the amount of the fimbriae or E. coli cells (competitor).

5. Inhibitor of the fimbriae-receptor binding assay:

(1). A series of concentration of the inhibitor in a buffer (50 ul/well) are added to the wells of a microplate.

(2). A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the inhibitor and the controls.

(3). The reactions between the inhibitor and the immobilized receptor for binding to fluo-fimbriae or between the inhibitor and the fluo-fimbriae for binding to the immobilized receptor are initiated by inserting the 96-pin lid coated with the receptor into the wells. After incubation at 37^0 C for 1 hr the 96-pin lid is taken out of the vessel to stop the reaction.

(4). The fluorescent intensity of the fluo-fimbriae remaining in the wells of the microplate is determined using a fluorometer and is directly proportional to the amount of the inhibitor (competitor)..

As can be seen, the above-described assay has a wide range of applications due to its versatility. Furthermore, the assay does not require washing or "stopping" steps which are time-consuming and can lead to loss of signal or contamination. It is of note that in the examples above, the probe comprises a pin-shaped member and the reaction vessels are wells of a microtiter plate. However, it is to be understood that the probe may be of suitable shapes and arrangements, as discussed above, as may the reaction vessels.

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.